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PARAMECIUM BURSARIA: LIFE HISTORY. I. IMMATURITY, MATURITY AND AGE[1]

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Introduction

The discovery of mating types or sex types in the ciliate infusoria has made it possible to breed and cross these Protozoa as readily as higher organisms. This has made possible under favorable conditions a renewed study of the problems of youth, age and death, particularly in relation to conjugation. These problems form the subject of the investigation of which a first installment is presented here.

The unit for examination in such studies is the clone rather than the single cellular individual. We deal with youth, age and death of clones, not of single cells only. The clone consists of all individuals derived by vegetative fission from a single ex-conjugant. The age of the clone is properly reckoned from the time of separation of the two ex-conjugants of the ancestral pair.

A number of investigators, beginning with Maupas (1888, 1889) have reported that there is, following the separation of the conjugants, a period of immaturity, during which multiplication by fission occurs, but conjugation does not occur. This is followed, according to these reports, by a period of maturity during which conjugation may occur. The period of maturity is said to be followed by a period of decline or degeneration, often spoken of as age or senescence. According to Maupas (1888) conjugation during this period of decline results in the death of the conjugants. The decline itself, without conjugations, also ultimately results in death. G. N. Calkins was long the outstanding representative of this general conception of the life history of the ciliate, though he did not, I believe, report that conjugation during the period of decline results in death.

However, a number of investigators have shown that in some of the ciliate infusoria and in certain other Protozoa, a clone may, under favorable conditions, continue indefinitely to multiply by fission without decline, degeneration or other indications of senescence (Woodruff, Metalnikoff, Bělař, Hartmann, Beers, Dawson and others). A detailed review of these investigations, with references to the literature, is found in Jennings, 1929.

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However, after these demonstrations as before, it continues to be observed that clones of ciliates, cultivated for long periods in the laboratory undergo gradual decline and ultimate degeneration and extinction. The graph of vitality, as indicated by the rate of fission, shows a gradually descending course, as illustrated for many cases in Jennings, 1929. The experience of the present writer in recent long-continued culture of *Paramecium bursaria* has been of this type.

What is the nature of this gradual decline and degeneration, in the cases where it occurs? What is its relation to environmental conditions, to ageing, to conjugation, to other developmental or genetic processes? It is with these and related questions that the present investigation deals. A method of detecting and following the progressive decline of clones was discovered in the fact that the mortality after conjugation commonly increases with the age of the conjugating clones. This has made possible a detailed study of the problems.

The fact that clones of *Paramecium bursaria* show, beginning immediately after separation of the conjugants, successive periods of immaturity, adolescence, maturity, and decrepitude in age, has been mentioned in previous papers (Jennings, 1939; 1939a; 1941; 1941a; 1941b; 1942). The phenomena have now been subjected to extensive cultural and experimental analysis. In presenting the results, the different periods of life will be successively taken up. Clones now or recently in the laboratory have been cultured for many different periods, up to eight years. Some of these clones are derived from individuals collected in nature; others were produced by conjugation in the laboratory. These numerous clones furnish abundant favorable material for examination of the pertinent questions.

The investigation has been devoted mainly to the genetic or intrinsic factors involved, though later installments will deal to some extent with the action of environmental factors. The method of work has been largely that of inducing conjugations within or between known clones, particularly at different ages, and following the history of the clones produced at these conjugations. Other results of such matings, particularly as to the inheritance of sex types or mating types, have been presented in detail in two previous papers (Jennings, 1941; 1942); those results form a foundation for much of the work to be presented in the series here begun.

The conditions in *Paramecium bursaria* that are particularly favorable for this type of work may be briefly recapitulated. Any variety of the species is differentiated into several mating types or sex types. One variety has eight sex types; two others have four each; a fourth variety seemingly has but two. In any clone all the individuals are of the same sex type, save in the infrequent cases of differentiation of a clone into two sex types (see Jennings, 1941). Mature clones of a given sex type conjugate readily with any of the other sex types of that variety (when their individuals are mingled together) but not with clones of their own sex type, nor with clones of other varieties. The readiness to conjugate and the mortality after conjugation vary with the laboratory age and with certain other conditions, as will be set forth.

The investigation has been an extensive and long-continued one. The author has been able to devote his time and energy to it exclusively, so that it has been the hope and design to devote so much time and attention to each problem that arose as to clear it up fully. This hope has been realized, I believe, for certain aspects of

the work, but also many questions have been raised that will require further work. The investigation deals with all the various periods of the life history of clones, but the chief problem for study has been age and natural death. The present first installment deals mainly, however, with the periods of immaturity and maturity, and with a general survey of the phenomena of ageing and death. Later installments will present detailed experimental investigations of ageing and death and of their relations to conjugation.

Culture Methods

The organisms were cultivated in the lettuce infusion impregnated with *Flavobacterium brunneum* (see Jennings, 1939; 1942). In the earlier years the alga *Stichococcus bacillaris* was added to the infusion (Jennings, 1939). In later years the alga was not used, as the organisms flourish equally well without it. Also for certain later years the infusion employed was diluted to one half the concentration described in 1939, but in the long run this does not work as well as the more concentrated infusion. For months the organisms flourish as well as they do in the concentrated infusion, but decline begins earlier in the dilute infusion. Therefore from 1943 the more concentrated infusion was used, as in the earlier years.

If the infusorians are under culture in a small vessel, such as a Syracuse dish, or in the deep depressions of thick culture slides, they continue to multiply vigorously if transferred to new culture fluid about once a week. If the cultures are left unchanged for considerably longer periods, some of them continue to flourish, but in most of them the animals cease to multiply after a time, and they become thin, at the same time decreasing in number. Why some old cultures continue to flourish, while most do not, is obscure. The infusoria may live in a depressed condition in the old cultures for long periods, reviving and becoming vigorous, with resumption of multiplication, when transferred to fresh infusion. There are indications that this environmentally-induced depressed condition is due in some considerable measure to the unfavorably altered condition of the infusion, possibly to deleterious products of metabolism from either themselves or the bacteria on which they feed, rather than to mere lack of food. In any case it is important to distinguish such temporary and purely environmental depressions from the depressed conditions of the clone that is largely independent of present environment, as described later. Whether however there is a connection between the two—whether long-continued extrinsic environmental depression may tend in time to induce intrinsic or genetic depression—may remain for the present an open question.

Clones differ greatly in their resistance to unfavorable cultural conditions. Some die out quickly under conditions in which others continue to exist and from which they recover when the conditions improve. Particular clones, indeed, differ at different periods of their lives in their resistance to unfavorable conditions: a matter which forms in large measure the subject of the present investigation.

The clones that have been cultivated for long periods in our laboratory have as a rule been subjected at intervals, particularly in the earlier years, to periods of environmental depression. This appears to be of importance in connection with the long periods of immaturity shown by some of the clones, as will be set forth in a later section.

Special methods will be described in connection with the phenomena in relation to which they are employed.

Immaturity: Its Dependence on Cultural Conditions

For a period of time after conjugation the descendants of the ex-conjugants neither form clots nor conjugate, even though brought under favorable conditions into intimate contact with individuals of different sex type, belonging to the same variety. This period of sexual immaturity varies greatly in length in different clones, even among those produced by conjugation of the same two parental clones. Immature periods varying in length from a minimum of twelve days up to some years have been observed (see Jennings, 1939, pp. 214–215). There is among our clones one ("403*a*1") that was produced October 20, 1937 (by self fertilization of a clone of Variety I); it still does not conjugate (July 1943) though more than five years old. This clone that has never become mature has a well developed micronucleus (T. T. Chen, personal communication); it has now (1943) passed into a period of depression.

The great variation in length of the period of immaturity, taken in connection with the fact that many or most of the clone cultures were subjected at times to the depressing conditions mentioned above, suggested that possibly the age at which sexual maturity is reached depends to some extent on the cultural conditions. This was tested in the following way:

By conjugation between two clones a large number of pairs was obtained. From these pairs were derived many ex-conjugant clones (two from each pair). Each of these ex-conjugant clones was divided, after it had multiplied to some extent, into two parts. One part was kept in vigorous multiplication by changing and renewing frequently the culture fluid, and by keeping the number of individuals small; this is known as the rapid portion (R). The other part of each clone was subjected to unfavorable conditions, which interrupted or made very slow the multiplication by fission; this is known as the slow portion (S). This was done by allowing the cultures to become crowded, and the culture medium to become old. There were thus genetically identical sets of R and S clones, with many clones in each set. After a period of this diverse culture, the two sets of clones were restored to the same favorable cultural conditions. Here they were allowed to multiply, and at intervals the clones of both sets were tested in the usual way to determine if they were sexually mature. That is, the two divisions (R and S) of each clone were tested each with the four mature sex types of the variety (Variety I). Clones that were mature reacted by forming clots and pairs with three of the four sex types; those that were immature did not form clots or pairs.

Two extensive experimental cultures of this type were carried through; both showed that the cultural conditions in which a clone has lived do indeed affect the age at which it becomes mature. Each experiment lasts for many months and requires several or many successive tests of each clone, at intervals of weeks or months.

The first experiment was begun May 9, 1941, at which time 24 pairs were obtained from the conjugation of the two clones 39 and 44, respectively of the sex types D and B of Variety I. (Clone 39 was collected December 23, 1939 at Monterey, California; clone 44 March 6, 1941, at Westwood Village, Los Angeles,

California.) After separation the two ex-conjugants of each pair were isolated, so that there were 48 ex-conjugant clones. Each was allowed to multiply separately in fresh culture medium until May 20 when a considerable number of individuals was present in each of 46 of the clones (two of the 48 ex-conjugant clones were non-viable).

Now each of the 46 ex-conjugant clones was separated into two parts: a "rapid" part, designated R and a "slow" part, S. There were now therefore 92 separate cultures, two for each of the 46 clones. Each "rapid" culture, R, was begun with but ten individuals, while its corresponding "slow" culture, S, included at the beginning a large number of individuals (the remainder of the clone). The rapid cultures were kept in vigorous multiplication; every second day four individuals of each were employed to carry on the culture, and these were transferred to abundant fresh culture medium, the rest of the culture being discarded. In the slow cultures of the 46 clones, on the other hand all the individuals were left in each case on the depression slide and were there allowed to multiply till they became so numerous that reproduction nearly or quite ceased and the individuals became thin. As the culture fluid evaporated, fresh weak fluid was added, at intervals of several days, but there was in these slow cultures no transfer to fresh slides or infusion. Thus the slow cultures were kept for a long period in a depressed condition. Their individuals remained normal in appearance, but were slender and did not become numerous.

At later periods both the rapid and the slow were placed in fresh fluid in Syracuse dishes, and were allowed to multiply there till they became sufficiently numerous to be tested for maturity. At the time of testing, therefore, the two sets were living under the same conditions and were both multiplying well. Does the earlier different history of the two make a difference in the age at which maturity is reached?

The first test was made June 6 to June 23, 1941, when the clones were about one month old. The first 12 clones were tested from each of the two sets of cultures, the tests being carried out in the usual way. None were yet mature; no clots or pairs were produced.

The next test was August 7 to 16, 1941. Five of the slow cultures had succumbed and died out; the corresponding rapid cultures of the same clones were discarded. This left 41 clones, each represented by a rapid and a slow culture. All those in the rapid cultures except four had now become sexually mature, as shown by their reaction (production of clots and pairs) with the test clones. But none of the slow cultures reacted; none was mature.

Thus the difference in cultural conditions had produced a great difference in the age at which the organisms became sexually mature. At the age of 3 months those parts of the 41 clones that had been kept vigorously multiplying were all mature except four, while their genetic duplicates that had been subjected to depressing conditions were all still sexually immature.

The four clones of the rapid set that did not react in the August tests became mature in October. At the age of 5 months all the 41 clones in their rapid cultures were mature.

From the time of the August tests the slow cultures were tested at intervals of about one month. Some additional cultures died out, so that 33 clones were tested

in the slow cultures in October; none were yet mature. In the latter half of November 1941, the slow cultures of 30 clones were tested, one had now become mature. No additional culture of the slow set had become mature at tests in December 1941, January 1942, February 1942, or March 1942.

In July 1942, 14 months after conjugation, the still surviving cultures of the slow set were again tested. Only eight clones were now represented in the eight living cultures of the slow set. Of these, four were now fully mature, while two others were becoming mature, since they reacted with two of the four sex types of the variety. The other two cultures were still immature.

Thus while clones kept in vigorous multiplication all become mature at the age of 3 to 5 months, the same clones subjected to depressing conditions became mature only at 10 to 14 months or later.

It appeared desirable to repeat this experiment. The second experiment began October 22, 1941, at which time were isolated 54 pairs, produced by the conjugation of two clones derived from two different pairs of the "rapid" set of the experiment just described. On October 27 each of the 108 ex-conjugant clones from these 54 pairs was divided into two parts, a "rapid" lot (R) and a "slow" lot (S). The rapid set was cultured, as in the previous experiment, in such a way as to keep the individuals multiplying rapidly. The slow set (duplicate of the rapid) was subjected, as before, to crowding and starvation. There were thus 108 clones in each set.

The cultures of the "slow" set were subjected to depressing conditions, in this case, for but 18 days, till November 14, 1941. On this date all the surviving clones, R and S, were placed in Syracuse dishes, and thenceforth all were cultivated in the same way, in the usual dilute lettuce infusion. The point to be determined is whether the cultivation of one of the duplicate sets under depressing conditions for 18 days, while the other set was vigorously reproducing, has made a difference in the age at which sexual maturity comes on.

In this second generation experiment the mortality of the clones was much greater than in the previous generation. Of the total 216 ex-conjugant cultures (sum of the R and the S), only 91 survived to be tested. These included the same clone in both sets in 37 cases (37 R and 37 S that were genetically identical). In addition there were 5 clones that were represented in the R set but not in the S set, while 12 other clones were represented in the S set, but not in the R set. That is, 42 clones were represented in the R set, 49 in the S set, and 37 of those in each set were the same clones.

In the 37 clones that were common to the two sets, the rapid culture became mature in every case before the slow culture of the same clone. In 22 of the 37 cases only the rapid culture became mature within the 8 months during which the experiment was continued, the same clones in the slow cultures remaining immature to the end. In the other 15 clones of the 37 that were represented in both sets of cultures, both cultures became mature, the rapid culture in every case a month or more earlier than the same clone in the slow set.

Up to the end of February 1942, when the clones were a little more than 4 months old, 42 clones of the R set had been tested, and 36 were found to be mature; that is, in the rapid cultures 85.7 per cent of the clones were mature at the end of 4 months. At the same date 46 of the 49 clones in the slow set had been tested, of

which 14 were found to be mature, so that but 30.4 per cent of the clones in the slow cultures were mature at the age of 4 months. At the end of June, when the clones were 8 months old, 40 clones were mature of the 42 in the rapid cultures (95.2 per cent) while at the same time but 20 of the 49 clones in the slow cultures were mature (40.8 per cent).

Thus these two long-continued experiments give the same result. Clone cultures that are kept vigorously multiplying, without starvation or crowding, or staleness of the culture medium, become mature much earlier than the same clones in cultures that have been subjected for a considerable period to the depressing effects of the conditions just named. Those kept vigorously multiplying usually become mature in 3 to 4 months, though there is much variation among them, and in some the immature period is much shorter. Those that have been subjected to depressing influences such as starvation, crowding and staleness do not become mature till they are 10 to 14 months of age, or older. In both the experiments described above a number of the clones thus subjected to depressing conditions did not become mature during the time the experiments continued.

In these phenomena we see temporary differential action of diverse environments producing in the stocks differences that continue for months of vegetative reproduction after the differential environmental action has ceased. In the second experiment described above the effectively diverse environmental conditions lasted for but 18 days, but the two sets thus diversely acted on retained the induced diversity for at least 7 months. The induced diversity is transmitted in vegetative reproduction for many successive generations.

It has been suggested verbally to me that possibly the time of becoming mature depends on the number of vegetative generations that have passed since conjugation. Depressing conditions decrease the number of vegetative generations passed through in a given period of time, and would therefore, if the above suggestion is correct, lengthen the time to maturity. But in view of the great difference in the time of becoming mature induced by a relatively short period of depressing conditions, the above suggestion appears not probable, and it is more likely that the action of depressing conditions is more direct, producing physiological changes that delay the attainment of maturity more or less independently of the number of vegetative generations that have passed. The matter is one that is worthy of precise and detailed experimental study, as indeed is the entire phenomenon of the relation of the attainment of maturity to environmental conditions.

The facts shown by the experiments are of practical importance for the investigator. For many purposes it is necessary to cultivate the ex-conjugant clones until they are mature, in order that needed crosses may be made. The time required for this is greatly shortened if the clones are kept continuously in vigorous multiplication. It appears probable that the frequent very long periods of immaturity in my own work mentioned in earlier papers, were due to the fact that in their culture the clones were at times subjected to depressing conditions.

There is an important additional relation observed on comparing the rapid and slow subdivisions of the clones. Though clones produced by different pairs are frequently of different sex types, the two subdivisions of any single ex-conjugant clone are always finally of the same sex type, in spite of the fact that they have been cultured differently and have become mature at widely different periods. In the

two experimental cultures of which account is given above, there were 21 ex-conjugant clones in which the sex-type was determined for both the rapid or early maturing, and the slow or late maturing subdivisions. Of the 21 ex-conjugant clones 13 were of sex type A, five were B and three were C. In all cases both subdivisions of any clone were of the same sex type. This agrees with all other evidence in showing that the sex type is determined genetically and is not ordinarily altered by changed environmental conditions.

Transitional Period from Immaturity to Maturity

In the life of most clones there is a period of transition, during which the ex-conjugant clone reacts sexually only in sporadic individuals, and in some cases with only one or two of the sex-types of the variety to which it belongs. This transitional period may last for weeks. Details, with many examples of the sporadic sex reactions, have been given in the third report on these investigations (1942, pages 196–199). Rarely the transitional period is short, or possibly entirely lacking, the clone suddenly acquiring the typical strong reaction in all (or most) of its members, with the usual clot formation and resultant numerous pairs.

Maturity

The sexually mature period is characterized in typical cases by the fact that when clones of different sex type are mingled, there occurs the strongly marked clotting followed by formation of numerous pairs (Jennings, 1939).

It is notable, however, that the strength of the tendency to clot varies greatly in different clones. Some clones when mixed form at once large clots, like those photographed in the 1939 paper. Others form but small clots, containing only a few individuals (three or four or less). In some clones only a few individuals take part in the clotting, while in others all are active in the sexual reaction. Some clones do not react at once when mixed, but do react later. Some do not react on the day the mixture is made, but react (strongly or weakly) the next day.

These differences in the tendency to clot resemble those described by Moewus in certain flagellates, which Moewus has correlated with different genetic constitutions. (For summaries of this work of Moewus, the paper of Sonneborn, 1941, may be consulted.) These phenomena are worthy of detailed study in the ciliates.

The period of sexual maturity lasts for several years, and is followed by a period of decline which forms the chief subject of the present investigation.

A remarkable phenomenon is to be observed at times in mature clones. When two clones of different sex type (but of the same variety) are mingled, strongly marked clotting usually occurs, the individuals coming into intimate contact; but in some cases no conjugated pairs are finally produced. The clotting occurs during the day; toward evening the clots break up, not into pairs, as in the normal case, but into separate single individuals. In the normal case, after clotting has occurred, many united pairs are found to be present in the mixture the next morning, before the new clotting of the second day has begun. These pairs remain in union for 36 hours or more. But in exceptional cases no pairs are present the next morning. On the second day clotting may occur again, but as before no lasting pairs are

formed. Thus the first stages of the mating reaction occur, but the final stages do not; conjugation is not completed.

A similar phenomenon has been described by Sonneborn (1942) in certain clones of *Paramecium aurelia*. In *Paramecium bursaria* such clotting without formation of pairs may at times characterize many clones of a collection. Such cases are the following: In February 1943 collections were made from ponds in the Botanical Garden of the University of California at Los Angeles; also from ponds in the Municipal Park at Beverly Hills. A considerable number of these clones showed clotting without formation of lasting pairs, presenting opportunity for a study of the phenomena.

From the two collections 46 clones were isolated and cultivated. These included representatives of all the four sex-types of Variety I. Three clones were of sex type A, nine of type B, 24 of type C, and ten of type D. Representatives of each clone were tested with all clones of the three types to which the clone under test did not belong. In all cases clots were formed, but a considerable number of the mixtures did not form lasting pairs. All clones of sex types A and D (13 clones in all) formed clots and pairs in the normal way, when mixed with clones that were capable of forming pairs. Of the nine clones of the B type, four were normal, forming clots and pairs in the usual way, while five ordinarily did not form pairs, even when mixed with clones that were themselves thoroughly normal. All these form clots as usual, though no pairs. Of the 24 clones of C type, seven formed clots and pairs in the usual way, while 17 formed clots but did not form pairs. Thus of the 46 clones 24 formed clots succeeded by pairs in the usual way, while 22 clones formed clots but did not form pairs (save in isolated instances; see next paragraph).

A peculiar feature of the phenomena is that certain of the clones that as a rule do not form pairs may in certain instances form one or two pairs. This occurred in mixtures in which in normal reactions there would be found as many as a hundred or more pairs. Such isolated pairs were observed in five of the clones that commonly formed no pairs. Three of them were of type C, while two were of type B. In each of four of these five clones but a single lasting pair was observed, though there were for each clone several mixtures in each of which many pairs would in the normal case be formed. In the fifth clone ("BH130") no pairs were formed in mixtures with any of the 37 clones of different sex types that belong to these collections; but three pairs were formed when individuals of this clone were mixed with certain "tester" clones (of types A and D), that had been selected because of their strong sexual reactions.

To determine whether a micronucleus is present in the clones, particularly in those that do not form pairs, a cytological examination was made by Dr. T. T. Chen. This examination covered 41 of the 46 clones in the collection mentioned above; it included 19 of the clones that do not ordinarily form pairs, and 22 of those that do. A micronucleus was found to be present in all the clones that regularly form pairs; also in 15 of the 19 clones that clot but do not form pairs. Four clones were without micronuclei, and all of these belonged to the group that clot but do not ordinarily form pairs. Two of them had produced an isolated pair or two in certain mixtures.

Absence of a micronucleus thus usually, though not always, prevents pair forma-

tion, though it does not prevent clotting. But other conditions may prevent formation of pairs, since 15 clones that had micronuclei did not form pairs.

The absence of a micronucleus does not prevent the manifestation of the sex type. Of the four clones without micronuclei, three belonged clearly to sex type B, while the fourth was a well defined type C.

In the other 37 clones the micronuclei varied considerably in form, size and stainability. But no special characteristics appeared peculiar to the 15 clones that did not form lasting pairs.

The cytological changes in this clotting without conjugation, or "formation of temporary pairs" have been examined by Dr. T. T. Chen, who will report on them in a separate paper.

Age, Mortality, and the Consequences of Conjugation

We have in the laboratory many clones of the different varieties of *Paramecium bursaria*. In January 1943 there was under culture a reserve stock of 264 clones, in addition to many recent ex-conjugant clones that were under immediate study. The clones are of many different ages. Some have been under cultivation for about eight years (April 1943). Others vary from six years to but a few months or days of laboratory cultivation. Many of the clones were collected in nature, from diverse parts of the United States or abroad. Others were produced in the laboratory by the conjugation of pre-existing clones.

Many of these clones that at first flourished vigorously have since declined in vigor, with alteration of many of their life phenomena. Some have died out, in spite of the greatest care in cultivation. Many of the old clones at conjugation produce pairs of which the majority die. These observations formed the starting point of the present investigation.

Symptoms of Degeneration as Clones Become Old

Our oldest clones, Nos. 1 (or "m") and 2 (or "l") were collected April 18, 1935, at Alexandria, Virginia. At the time they came into my hands, in June 1937, they had been maintained for two years in the laboratory by Dr. T. T. Chen. What their age may have been at the time of collection there is of course no way of knowing, so that the total age of each is unknown. The two have shown in the later years depression or degeneration manifested in a number of different ways. Their history is instructive.

When they came into my hands in June 1937 Clones 1 and 2 were vigorous in vegetative reproduction, but showed a high mortality at the time of conjugation. The two were of different sex type (No. 1 of type C, No. 2 of type A), and were bred together in June and July 1937. From their mating 142 pairs were obtained, yielding, after separation of the conjugants, 284 ex-conjugants. Of these, descendants of but 18 ex-conjugants survived and formed clone cultures; that is, but 6.3 per cent of the ex-conjugant clones survived.

The clone No. 1 was divided into many cultures which were cultivated separately. In January 1938 certain of these cultures yielded pairs by self differentiation and self fertilization. There were 118 of these pairs from the selfing of clone Number 1, yielding 236 ex-conjugants. Again the mortality was excessively high,

only six of the 236 surviving and forming ex-conjugant clones. Thus but 2.5 per cent of the ex-conjugants from the selfed clone No. 1 survived.

Not all of the cultures No. 1 underwent self differentiation and self fertilization. Those that did so contained in consequence individuals of the two mating types C and B, which conjugated, yielding pairs as just set forth. But several cultures remained of the pure original type C. In order to insure that the cultures should contain only this type, new cultures were started from single individuals, of the mating type C.

In February 1940 it became apparent that the cultures of clone No. 1 were less flourishing than those of other clones. By October of that year all cultures of this clone had become scanty, and when new cultures were seeded with a number of individuals of the clone there was little multiplication. The contrast with other clones in this respect was striking. Individuals of abnormal form made their appearance in the cultures of clone No. 1. A number of the cultures died out.

From October 17 to October 28, 1940, a period of 12 days, comparative isolation cultures were carried on of this clone No. 1 and of three other clones (see Table I). These were for the purpose of comparing the rate of fission and the

TABLE I

Paramecium bursaria; rates of multiplication and frequency of deaths in certain clones, in comparison with Clone 1, for the period October 17 to October 28, 1940.

Each clone consists of 24 parallel lines cultivated for 12 days. The number of fissions in the 24 different lines during the 12 days is summarized; also the total number of deaths of lines in each clone of 24 lines.

Clone 1 (Laboratory age 66 months). Number of fissions in the different lines varies from 0 to 7. Number of deaths, 14.

Clone 2 (Laboratory age 66 months). Number of fissions, 17 to 23. Number of deaths, 4.

Clone 6 (Age 40 months). Number of fissions, 20 to 27. Number of deaths, 2.

Clone 36 (Laboratory age 11 months). Number of fissions, 27 to 30. Number of deaths, 1.

frequency of deaths in clone 1 with those of the three other clones. Twenty-four lines of each clone were cultured on depression slides. Each of the 24 lines of each of the four clones was begun as a single individual in one of the depressions of the slides. Each was allowed to multiply for 24 hours; then the number present was recorded and a single individual from each depression was transferred to a new slide and fresh fluid, and allowed to multiply for 24 hours as before. This was repeated for each of the 96 lines throughout 12 days. At the end of the 12-day period there were records for the 24 lines of each of the four clones. In Table I are given the number of fissions in the lowest and highest line of each clone; in other words, the range of variation in fissions for the 24 lines of the clone. Table I also gives the number of deaths in the 24 lines of each clone during the 12-day period. This number of deaths was obtained as follows: After the daily transfer of a single individual to a new slide, sometimes this individual died, ending the line. The line was then continued by substituting an individual from one of the other lines of that clone. The "number of deaths" in Table I shows how many times this occurred in the 24 lines of each clone.

The clones compared with clone 1 in Table I are the following:

Clone 2 was collected at the same time and place as clone 1, and hence was of

the same laboratory age. It was not vegetatively depressed at the time of the cultures of Table I.

Clone 6 was derived from a pair resulting from the conjugation of clone 1 with clone 2, June 18, 1937. It is therefore younger than its parent clones 1 and 2 by somewhat more than 2 years.

Clone 36 was collected at Los Angeles, November 18, 1939.

Thus some of the lines of clone 1 did not divide at all during 12 days, and the most vigorous line divided but seven times. In contrast, the other three clones, living under exactly the same conditions, multiplied at the rate of one, two, or more fissions daily, in each line. The clone 1 shows many deaths and hardly multiplies at all.

During November 1940 it became increasingly difficult to keep the cultures of clone 1 alive. There were many such cultures, some on slides, some in Syracuse dishes. Some were cultivated in the dilute lettuce infusion, some in the more concentrated, some with algae, some without. Under all these conditions other clones multiplied vigorously. But one after another the cultures of clone 1 died out, until on January 26, 1941 the last culture died and the clone 1 became extinct.

A similar downward course has been followed by certain other clones. Some have become completely extinct; others still exist as weak scanty cultures that are kept alive only with difficulty. All this has occurred under conditions in which other clones flourish. Some examples may be mentioned.

The clone "McD3" was collected near Baltimore, Maryland, February 7, 1938. It was long one of the most vigorous of the clones in the laboratory, and was employed as the chief tester of sex type M, Variety II. But it became weak and degenerate, and for many months in 1942 and 1943 it was kept alive only with difficulty. It finally died out, in all its cultures, in March 1943.

A considerable number of clones of Variety III were obtained, some from North Carolina, February 25 and March 20, 1938; some from Provincetown, Massachusetts, July 28, 1938. By crosses among the clones from North Carolina many additional clones were obtained. Some 24 clones were kept under culture as a reserve stock. Now after more than 5 years almost all of these stock clones have died out. Before final death they were for many months in bad condition, multiplying little and showing hardly any tendency to sexual reaction. The few clones that remain are in bad condition and will doubtless soon die. As Variety III is not known to occur on the Pacific slope and has rarely been collected elsewhere, the loss is a serious one, leaving the laboratory without testers for Variety III.

A peculiar variation in these histories is shown by the clone known as S of Variety II. It was collected in the spring of 1937. For years it flourished and was employed as a tester of the sex type J. In 1940 to 1943 it became very weak and many of its cultures died out. All of those which were kept rapidly multiplying died, but a certain old quiescent culture, in which the animals were thin and multiplied little or not at all, has remained alive. Attempts to bring its members to rapid multiplication by additions of fresh culture fluid results in their immediate death.

Many other cases of decline or degeneration of clones after they had long been under culture have been followed in this laboratory. A large number of stock cultures were long kept on hand. These were transferred weekly to new cultures.

For a long time they flourished, now more than half of them have died out (June 1943).

Age of Clones in Relation to the Readiness to Conjugate and to Fertility and Mortality in Conjugation

Observations on decline in old clones such as set forth above left in many cases the further impression that old clones may conjugate but that the mortality after such conjugations is greater than usual, many of the ex-conjugants dying without the production of long lived clones. Maupas in his great papers of 1888 and 1889 was left with a similar impression. He gives many instances of high mortality at conjugation in Stylonychia, in Onychodromus, in Leucophrys, in Didinium, in Spirostomum. In some of these cases the stocks were known to be old; in others this was uncertain. In some cases Maupas attributed the high mortality to the supposed or known fact that the conjugating animals were close relatives. In view of these impressions and the accounts given by Maupas, an extensive investigation was undertaken of the relation both of age and of inbreeding to mortality at conjugation. The results of this work will be presented in full in later papers.

Since the time of Maupas the only work bearing on this precise matter appears to be that on mortality at endomixis in *Paramecium aurelia*, carried out mainly by Sonneborn or under his inspiration, and published by Jennings and Sonneborn, 1936, by Gelber, 1938, and Pierson, 1938. At the time the work was done it was not known that "endomixis" is a form of conjugation, or closely related to it, as "autogamy." But Diller (1936) showed for *Paramecium aurelia* that in "endomixis" two nuclei unite, both produced of course by the single individual, so that the process is a close form of self-fertilization; and this was confirmed genetically by Sonneborn (1939). (Whether in all cases endomixis includes such autogamy is extensively discussed by Woodruff, 1941.) Jennings and Sonneborn (1936) showed that lines which omit endomixis die, and that many die at the occurrence of endomixis; also that "It appears that the longer endomixis is omitted the greater the proportion of individuals that die when they undergo it" (p. 419). This relation to the time elapsed since the foregoing endomixis was examined statistically by Gelber (1938) and Pierson (1938). They showed that the older the lines, reckoned from the last endomictic period, the greater the proportion of deaths at endomixis. Comparison of their results with those on conjugation in *Paramecium bursaria* will be presented in later installments of the present investigation.

Readiness to conjugate in old clones: In clone No. 1, above described, it was found that the individuals were ready to conjugate long after the time when the clone had become weak; it continued till very shortly before the death of the clone. In mixtures of clone No. 1 (sex type C) with clones of types A, B and D, clotting occurred January 5, 1940; though all cultures of clone No. 1 died out January 26 of that year. It was notable, however, that though typical clotting occurred, in but few cases were pairs formed and conjugation completed. From the abundant clotting of January 5 but four firmly united pairs persisted till the next morning. Three of them separated that morning, indicating that conjugation had not been consummated. The fourth separated at the normal time, later, but of the two ex-conjugants one died without fission, the other divided but once, then both its descendants died.

Other old clones formed clots and later pairs, when mixed with clones of other sex types. The fate of these pairs is to be taken up later.

Thus old clones of Variety I undergo clotting and pairing with other sex types up to a short time before their death from age; a result that agrees with the observations of Maupas on other species.

Age in relation to mortality at conjugation: In most epidemics of conjugation there is a certain amount of mortality among the ex-conjugants or their immediate descendants. When the clones that conjugate are old the percentage of mortality is high, as seen in certain instances cited above.

The relation of mortality at conjugation to age of the conjugating clones presents opportunity for experimental study of the nature and progress of ageing. It has therefore been subjected to extensive and intensive investigation. The data on this and the conclusions to be drawn are to be presented in papers soon to appear.

Summary

The life history of clones of *Paramecium bursaria* shows successive periods: (1) a period of sexual immaturity, during which sexual reactions and conjugation do not occur: (2) a transitional period during which weak sexual reactions occur in a few individuals: (3) a period of maturity, in which sexual reactions are strongly marked and the individuals conjugate readily: (4) a period of decline, ending in many (or all?) cases in death.

The length of the period of immaturity and the time of attainment of maturity depend on the cultural conditions. If the animals are kept rapidly multiplying, under the best of nutritive conditions, maturity comes on early; if they are subjected to periods of starvation or other depressing conditions, maturity comes on much later or not at all.

Ex-conjugant clones that are kept vigorously multiplying become mature in most cases at the age of three to five months, though cases have been observed of much earlier maturity, the earliest observed age of maturity being 12 days.

Ex-conjugant clones subjected for some time to depressing conditions become mature (even after restoration to favorable conditions) only at the age of 10 to 14 months. Certain clones have lived for years without becoming mature.

If single ex-conjugant clones are divided into two cultures, one subjected to conditions favorable to rapid multiplication, the other to unfavorable conditions, the two parts show these same differences. The part kept under favorable conditions matures months before the other part.

Subjection to depressing conditions for but short periods (18 days) delays maturity for months.

Thus temporary differential action of diverse environments produces in clones differences which persist through months of vegetative reproduction.

In the period of maturity, clotting and conjugation *en masse* are commonly produced when cultures of clones of different sex type are mixed. But in certain clones clotting occurs without the completion of conjugation; dense clots occur, but no pairs are formed.

The period of maturity lasts for several years. It is followed by a period of decline. In this period fission becomes slower; abnormalities appear; many individuals die, so that the cultures become scanty and finally die out completely.

Clones have been cultivated in the laboratory five to eight years, finally showing degeneration and death.

During the period of decline conjugation may occur up to near the very end. But conjugation of aged stocks results in the death of most or all of the ex-conjugants.

The relation of age to mortality at conjugation presents many features of interest, and gives opportunity for study of the nature and progress of ageing. This matter is to be presented in later contributions.

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